Association of osteopontin gene haplotypes with nephrolithiasis

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Osteopontin (OPN) is one of the glycosylated phosphoproteins produced in the kidney that can modulate nephrolithiasis. We had previously found a modest association between *OPN* gene polymorphisms and the risk for urinary stone formation. In order to determine if sequence variants within the *OPN* gene could be linked to the risk of nephrolithiasis; we sequenced the entire *OPN* gene of 45 stone forming patients and 54 control patients of Japanese ancestry. We identified 61 polymorphisms and of these evaluated four haplotype-tagging single nucleotide polymorphisms in a total of 126 kidney stone cases and 214 healthy individuals; all of Japanese ancestry. There was a significant association of two of these haplotypes located in the OPN promoter with the relative probability of nephrolithiasis; one of increased and one of reduced risk. Our findings provide potential support for significant increased and decreased associations between *OPN* gene haplotypes and the relative potential of stone formation in the Japanese population. We suggest that such genetic findings may help to clarify the function of OPN in nephrolithiasis.

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Nephrolithiasis is a complex multifactorial disease resulting from an interaction between the environmental and genetic factors. There may be as high as a 5–10% lifetime risk of developing nephrolithiasis.[1] When the kidney performs its important function of conserving essential nutrients, eliminating waste products, and excreting salt and water in the final urine, there is the risk of crystals formation owing to tubular fluid supersaturated with calcium salts, and crystals are frequently found, even in the healthy urinary tract. At the same time, the kidney also generates numerous macromolecular proteins, which have been isolated and identified in both the urine and kidney stones matrix over the years.[2–5] These proteins exist widely both in healthy individuals and in those with nephrolithiasis and modulate crystal nucleation, growth, aggregation and adhesion to renal epithelial cells, and greatly affect the risk of forming nephrolithiasis.[5–8] Complex interactions between crystals and macromolecular proteins in renal tubules play a key role in the pathogenesis of nephrolithiasis. Stones may arise from abnormalities of compromises in these interactions.

Osteopontin (OPN), a multifunctional glycosylated phosphoprotein, is a member of the SIBLING (small integrin-binding ligand, N-linked glycoprotein) family. OPN was not only identified as the main organic component in the urinary stones matrix, but also had remarkably increased expression in the distal tubular cells in the stone-forming rat.[2,9] An analysis of gene expression using microarray technology has shown that the *OPN* gene was markedly upregulated in rats during the development of calcium stone formation.[10] Many studies suggested the OPN was a natural inhibitor of abnormal calcification in the kidneys of rats and has a vital inhibitory role in the phases of stone formation, including crystallization, crystal retention, crystal congregation, and stone formation *in vitro* or *in vivo*.[11–13] Other studies have also shown that OPN plays a role in stimulating the deposition and adhesion of crystals to cells in the early stages of stone formation[14] and calcium oxalate monohydrate crystal coating with OPN correlated with increased adhesion tendency.[15] Whether it acts as an inhibitor or promoter of kidney stone formation is still controversial.

Understanding the genetic basis of complex human diseases has been increasingly emphasized as a means of achieving insight into disease pathogenesis. A single nucleo-

tide polymorphism (SNP) of the human *OPN* gene had been reported to be associated with kidney stone risk using a fluorescent 5′endonuclease assay by our study group.[16] Taking into consideration that the *OPN* gene is among the most promising susceptibility genes for nephrolithiasis, we resequenced the entire human genomic *OPN* gene in nephrolithiasis cases and controls, searched haplotype-tagging SNPs (htSNPs), and analyzed the association of haplotypes with the risk of nephrolithiasis in this study.

RESULTS

Human *OPN* gene polymorphisms

Approximately 11 kb of the *OPN* gene was entirely resequenced in 45 cases and 54 controls. A total of 61 polymorphism loci, including six novel polymorphisms, were validated and detected. One SNP, rs5860110, was removed from further statistical analysis because of deviation from the Hardy–Weinberg equilibrium ($P<0.001$). Twenty-three SNPs with minor allele frequencies $\geqslant 1\%$ (Table 1), including six novel SNPs, were used to test the allele frequency differences and calculate the degree of linkage disequilibrium (LD) (D′ and r^2) between these pairs of SNP loci. The position of the transcription start site was designated as $+1$.

LD and haplotype analysis

Linkage analysis was performed for all possible pair-wise combinations of 23 SNPs with minor allele frequencies $\geqslant 1\%$. Figure 1 shows a visual depiction of the degree of LD. The value of the standard disequilibrium coefficient D′ is used to assess the degree of LD. Four LD blocks were defined and a total of 11 htSNPs were detected (Figure 1, Table 1). Block 1, containing two htSNPs (rs2728127 and rs2853744) located in the promoter of the *OPN* gene; block 2, containing four htSNPs (novel 2, novel 3, rs2853749, and rs2853750) spanning the proximal promoter and intron 1; block 3, containing two htSNPs (novel 4 and novel 5) located in intron 3; and block 4, containing three htSNPs (rs6532039, rs4754, and rs1126616) located in a relative long region from intron 3 to exon 7. Neighboring blocks showed strong LDs toward each other. The genotypic distributions of the haplotypes generated from htSNPs in each block did not deviate from the Hardy–Weinberg equilibrium.

Association analysis

At first, we tested association of *OPN* gene polymorphisms and haplotypes in four blocks with kidney stone risk between 45 cases and 54 controls. Two novel SNPs, novel 2 and novel 3, were revealed to have a significant allele frequency difference between cases and controls ($P<0.01$) (Table 1). In block 2, four htSNPs generated four main haplotypes and significant differences of haplotype frequencies were detected in haplotype T-G-T-G (odds ratio (OR) = 0.0526; 95% confidence interval (CI): 0.007–0.402; $P=0.0001$) and haplotype G-T-T-G (OR = 3.7947; 95% CI: 1.31–10.989; $P=0.0093$) between cases and controls; whereas, there was no statistically significant association of haplotype frequencies in blocks 1, 3, and 4 (Table 2). Then, we further genotyped the four htSNPs in block 2 in an additional 76 kidney-stone cases and 160 controls. A similar analysis was

Table 1 | Association analysis of 23 SNPs with minor allele frequencies greater than 1% between 45 cases and 54 controls

No.	refSNPID	Position	Location	Alleles Major/minor	Case (*n*=45)	Control (*n*=54)	OR	95% CI	χ^2	*P*-value
2	rs2728127	−1747	Promoter	A/G	75/15	85/23	0.739	0.340–1.520	0.678	0.410
4	rs2853744	−615	Promoter	G/T	75/15	85/23	0.739	0.340–1.520	0.678	0.410
5	rs11730582	−442	Promoter	C/T	47/43	66/42	1.438	0.816–2.533	1.583	0.208
7	novel.1	−301	Promoter	A/C	86/04	104/04	1.209	0.294–4.978	0.069	0.792
10	novel.2	−145	Promoter	T/G	76/14	103/05	3.795	1.310–10.989	6.755	0.009**
11	novel.3	−144	Promoter	G/T	76/14	103/05	3.795	1.310–10.989	6.755	0.009**
19	rs2853749	953	Intron 1	C/T	75/15	82/26	0.631	0.311–1.281	1.641	0.200
20	rs2853750	1019	Intron 1	A/G	75/15	84/24	0.700	0.342–1.433	0.958	0.328
22	rs11728697	2079	Intron 3	C/T	42/48	60/48	1.429	0.815–2.505	1.553	0.213
25	novel.4	2557	Intron 3	T/A	70/20	82/26	0.901	0.464–1.751	0.094	0.759
26	novel.5	2571	Intron 3	A/T	65/25	80/28	1.099	0.585–2.065	0.086	0.770
27	rs6839524	2670	Intron 3	C/G	62/28	73/31	1.064	0.576–1.963	0.039	0.844
29	rs6840362	3056	Intron 3	C/T	86/04	103/05	0.958	0.250–3.680	0.004	0.950
32	rs6532039	3496	Intron 3	A/G	54/32	68/40	1.007	0.561–1.811	0.001	0.980
33	novel.6	4084	Intron 3	C/T	86/04	107/01	4.977	0.546–45.351	2.469	0.116
37	rs7695531	5014	Intron 5	A/G	87/03	105/03	1.207	0.238–6.131	0.052	0.820
38	rs6532040	5332	Intron 5	A/G	87/03	105/03	1.207	0.238–6.131	0.052	0.820
40	rs6811536	5535	Intron 5	C/T	87/03	105/03	1.207	0.238–6.131	0.052	0.820
44	rs4754	5822	Exon 6	C/T	60/30	68/40	0.850	0.473–1.529	0.295	0.587
47	rs10516799	6320	Intron 6	C/G	87/03	105/03	1.207	0.238–6.131	0.052	0.820
51	rs1126616	6982	Exon 7	T/C	60/30	68/40	0.850	0.473–1.529	0.295	0.587
54	rs1126772	7315	Exon 7	A/G	53/37	66/42	1.097	0.620–1.942	0.101	0.751
56	rs9138	7471	Exon 7	C/A	59/31	68/40	0.893	0.498–1.602	0.143	0.705

CI, confidence interval; htSNP, haplotype-tagging single nucleotide polymorphism; OR, odds ratio; SNP, single nucleotide polymorphism.
The boldface represents htSNPs. *P* values were calculated using the χ^2 test.
** $P<0.01$.

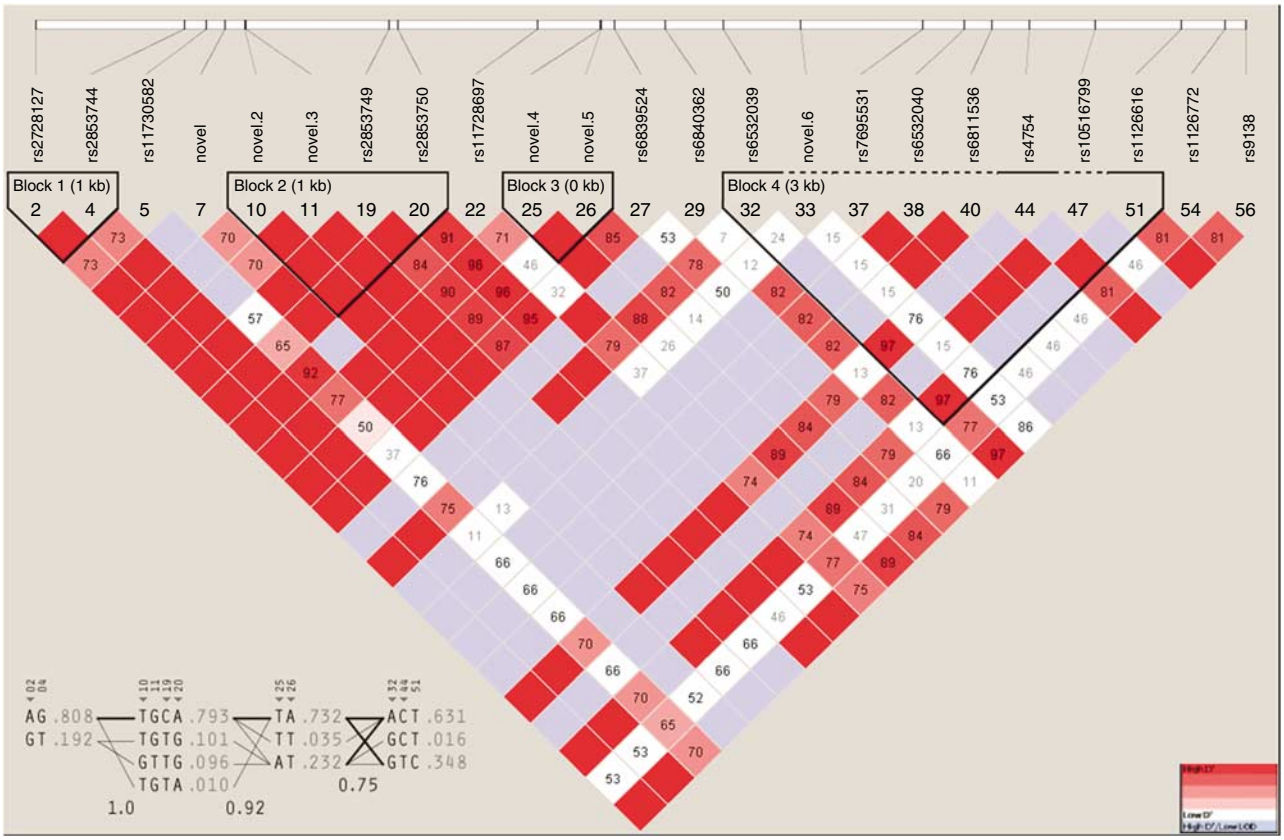

Figure 1 | Pair-wise LD and haplotype block structure in the *OPN* gene from 99 study subjects. The degree of LD was evaluated between any two SNPs with rare allele frequencies $\geqslant$1%. The white bar indicates the approximate span of the *OPN* gene. The corresponding number of the SNP is displayed in the upper triangle. The upper four pentagons represent the haplotype blocks and the boldfaced SNPs in the blocks represent htSNPs. Bottom left panels display the frequency of haplotype and levels of recombination between the blocks. The level of LD is indicated with the color scheme (bottom right).

Table 2 | Association analysis of haplotypes in four LD blocks between 45 cases and 54 controls

Block	Haplotype	Frequency	Frequency of haplotype		OR	95% CI	χ^2	*P*-Value
			Cases (n=45)	Controls (n=54)				
Block 1	SNP2-4							
	AG	0.808	0.833	0.787	1.3529	0.658–2.782	0.678	0.4101
	GT	0.192	0.167	0.213	0.7391	0.360–1.520	0.678	0.4101
Block 2	SNP10-11-19-20							
	TGCA	0.793	0.833	0.759	1.5854	0.781–3.220	1.641	0.2003
	TGTG	0.101	0.011	0.176	0.0526	0.007–0.402	14.685	0.0001**
	GTTG	0.096	0.156	0.046	3.7947	1.310–10.989	6.755	0.0093**
	TGTA	0.01	0.000	0.019	—	—	1.684	0.1944
Block 3	SNP25-26							
	TA	0.732	0.722	0.741	0.91	0.484–1.710	0.086	0.7695
	AT	0.232	0.222	0.241	0.9011	0.464–1.751	0.094	0.7587
	TT	0.035	0.056	0.019	3.1176	0.590–16.471	1.975	0.1600
Block 4	SNP32-44-51							
	ACT	0.631	0.632	0.630	0.9689	0.544–1.727	0.001	0.9692
	GTC	0.348	0.322	0.370	0.8082	0.448–1.458	0.501	0.4792
	GCT	0.016	0.034	0.000	—	—	3.75	0.0528

CI, confidence interval; LD, linkage disequilibrium; OR, odds ratio.
P-values were calculated using a χ^2 test or Fisher's exact test.
**$P < 0.01$.

performed in 126 cases and 214 controls, and the association was validated. The G allele carrier for novel 2 or T-allele carrier for novel 3 had a 1.6- (OR = 1.642, 95% CI: 1.058–2.549) or 1.8-fold (OR = 1.762, 95% CI: 1.132–2.743) increased kidney stone risk compared with those of the T-allele carrier for novel 2 or G-allele carrier for novel 3 (Table 3). In block 2, the haplotype G-T-T-G carrier had a remarkably higher (OR = 1.676, 95% CI: 1.072–2.621) and

Table 3 | Association analysis of htSNPs in LD block 2 between 126 cases and 214 controls

Block 2 htSNPs	Alleles Major/minor	Cases (*n*=126)	Controls (*n*=214)	OR	95% CI	χ^2	*P*-value
SNP10	T/G	203/45	363/49	1.642	1.058–2.549	4.954	0.026*
SNP11	G/T	207/45	381/47	1.762	1.132–2.743	6.41	0.0113*
SNP19	C/T	197/55	328/100	0.982	0.681–1.418	0.213	0.644
SNP20	A/G	193/59	328/100	1.003	0.694–1.448	0	0.9886

CI, confidence interval; htSNP, haplotype-tagging single nucleotide polymorphism; OR, odds ratio; SNP, single nucleotide polymorphism.
*$P<0.05$.

Table 4 | Association analysis of haplotypes in LD block 2 between 126 cases and 214 controls

Block 2 (SNP10–20)	Haplotypes frequency	Cases (*n*=126)	Controls (*n*=214)	OR	95% CI	χ^2	*P*-value
TGCA	0.756	192.0/60.0	321.9/106.1	1.055	0.659–1.364	0.08	0.7777
GTTG	0.132	43.0/209.0	46.8/381.2	1.676	1.072–2.621	5.189	0.0227*
TGTG	0.088	11.0/241.0	49.2/378.8	0.351	0.179–0.689	9.978	0.0016**
TGCG	0.012	4.0/248.0	3.8/424.2	1.801	0.438–7.395	0.667	0.414

CI, confidence interval; LD, linkage disequilibrium; OR, odds ratio.
*$P<0.05$. **$P<0.01$.

the haplotype T-G-T-G carrier had a remarkably lower (OR = 0.351, 95% CI: 0.179–0.689) risk of developing nephrolithiasis compared with other haplotypes (Table 4). A case-only analysis was performed among nephrolithiasis patients. No significant association was found between SNPs or haplotypes and clinical characteristics, such as gender difference, familial history of stone disease, biochemistry of the urine, primary age of first incidence, stone number, stone frequency, and stone component (data not shown). We also searched transcription factor-binding sites of the promoter region of the *OPN* gene. However, there was no transcription factor within the sites SNP10 or SNP11.

DISCUSSION

There is growing evidence that macromolecular proteins may play important modulating roles in the development of nephrolithiasis. We are in the process of comprehensively evaluating the possible associations between sequence variants of these proteins and kidney stone risk. OPN is a remarkable multifunctional molecule involved in diverse biological processes, including inflammation, cell survival, and immune responses.[17] It is also a central player in modulating and interacting with crystals. In this study, we resequenced the entire 11 kb *OPN* gene, including all exons, introns, an approximately 2100 bp promoter and 3′-untranslated region using 99 DNA samples. This strategy allowed us to discover previously unreported polymorphisms and perform a faithworthy LD and haplotype structure analysis. We genotyped 61 polymorphisms in the *OPN* gene and six novel polymorphisms were identified. Two novel SNPs (novel 2 and novel 3) located in the promoter region of the *OPN* gene were significantly associated with kidney stone risk ($P=0.026$ and 0.0113, respectively). Two haplotypes, a reduced risk-associated haplotype (T-G-T-G) and an increased risk-associated haplotype (G-T-T-G), were detected to be remarkably associated with nephrolithiasis ($P=0.023$ and 0.0016, respectively). These findings suggest that the *OPN* gene is dually associated with the risk of nephrolithiasis and may have a different function in crystal formation during the development of nephrolithiasis.

Since our discovery of the OPN protein from the urinary stone matrix in 1992,[2,9] the controversial question of whether OPN inhibits or promotes kidney-stone formation has been debated continuously in recent years. Many groups have characterized its unique expression profile, but few have searched for variants associated with kidney stone risk. Here, we identified two distinct haplotypes associated with nephrolithiasis. Different association of genetic variation with disease had already been reported in other study on *DLG5* gene and inflammatory bowel disease.[18] To our knowledge, our finding is the first direct genetic evidence that OPN can increase or reduce the risk of stone formation and provides a potential genetic clue to clarify this question in the future.

A recent study showed that Randall's plaques, initially formed in the basement membrane of the thin limbs of the loop of Henle, extended to the vasa recta and interstitial tissue and finally to the papillae.[19] In these processes, crystal particles may stimulate an array of responses, inducing localized injury and tubulointerstitial inflammatory reactions,[20] and macrophages are induced to release inflammatory cytokines, such as tumor necrosis factor-α and interleukin-6, and remove crystal deposits from the renal interstitium.[21] The OPN protein was observed to locate in cells of the loops of Henle and collecting ducts as well as on sites of plaque, mainly on the surfaces of the apatite crystal phase and at the junction of the crystal/organic layers.[22] Crystals and oxalate ions may be involved in the upregulation of OPN in renal fibroblasts.[23] As a multifunctional protein, OPN is involved in the inflammatory process[24] and the initiation of cell-mediated immune reactions,[25] regulation of inducible nitric oxide synthase in both macrophages and renal tubular epithelial cells,[26,27] protection of renal epithelial

cells from apoptosis,[28] and modulation of macrophage adhesion,[29,30] migration,[29,30] and cytokine release.[31] OPN is also associated with tissue repair and fibrosis.[32] The exact molecular mechanism by which OPN exerts its various effects in stone formation remains unclear. In addition, as a member of the SIBLING family, OPN was also reported to be coexpressed in the renal distal tubules along with its partner matrix metalloproteinase-3 in monkey kidney.[33] Interactions between OPN and matrix metalloproteinase-3 in the development of stone formation remain unclear. The fact that OPN knockout mice do not spontaneously develop crystal deposition indicates that other urinary macromolecular proteins can adequately compensate for the absence of OPN.[34] These observations may also add to the complexity of the stone-formation process. Definitive data regarding the role of OPN were lacking until recently.

Mo *et al*. reported that OPN was markedly induced in renal epithelial cells of Tamm–Horsfall protein-knockout mice and was moderately induced in wild-type mice under high calcium/oxalate conditions.[5] This finding suggests that OPN and Tamm–Horsfall protein may function together to keep renal stones from forming. The finding[35,36] that the concentration of OPN was lower in stone formers than those in healthy controls suggests that low levels of OPN may provide limited inhibition of crystallization. Our finding suggests, on a genetic level, that there might be different unknown pathogenesis between individuals with reduced risk-associated OPN haplotype and individuals with increased risk-associated OPN haplotype. Whether individuals with different haplotypes, under certain conditions, tend to express different OPN–protein concentrations remains unclear. These definite conclusions will be validated by a larger number of studies in the future.

The SIBLING family is characterized by similarities in gene structure, location as a cluster on the same chromosome 4 in humans, and expression in mineralized tissues.[37] Whether there exists high genetic LD among these genes remains unclear. Recently, other members of the SIBLING family, such as bone sialoprotein, dentin matrix protein-1, dentin sialophosphoprotein, and matrix extracellular phosphoglycoprotein, had also been observed to express or coexpress with its partner in normal monkey kidney tissue.[33] However, there has not been a study of the association between stone formation and the SIBLING gene family. In this study, we only focused on OPN as a candidate gene because of its unclear role in stone formation. Additional studies of genetic linkage of the SIBLING family will further clarify interactions among these genes in the process of stone formation.

In this study, several characteristics and limitations should be mentioned. First, our study individuals all came from the Asian Japanese ethnic group. This eliminated the possibility of confounding owing to population stratification. On the other hand, it also limited the ability to perform cross-population comparisons. In addition, the fact that the incidence of nephrolithiasis in Japan (about 3.8–9%)[38] is lower than that in Europe and North America (about 8–15%)[1] may not only reflect a difference in dietary habits including fluid intake, calcium and oxalate metabolism, and dietary fat intake, but also imply the possibility of distinct genetic polymorphism distributions in different ethnic groups. Second, an entire resequencing containing the non-coding region brought us unabridged variant information in *OPN* gene and provided us with a reliable analysis of the LD characteristics and haplotype block structure. Therefore, we believe that these findings represent true association in the Japanese ethnic group.

In summary, our study showed a dual association between OPN sequence variants and kidney stone risk and presents a potential genetic clue towards the role of OPN in nephrolithiasis.

MATERIALS AND METHODS

Study subjects

Our study group consisted of a total of 126 unrelated Japanese patients (mean age $\pm$ s.d. $= 57.6 \pm 13.5$ years) with nephrolithiasis, who underwent treatment at Nagoya City University Hospital from 2000 to 2006. The clinical characteristics of all of the patients are listed in Table 5. Blood and 24-h urine were collected after the patient's symptoms were ameliorated. Urine calcium, oxalate, and

Table 5 | Characteristics of 126 kidney stone cases

Characteristics	Cases (n)	%
Sex		
Male	93	73.8
Female	33	26.2
Primary age		
<=55 years	83	65.9
>55 years	43	34.1
Familial history		
Yes	9	7.2
No	117	92.8
Urine calcium excretion[a]		
Hypercalciuric	21	16.7
Normocalciuric	105	83.3
Urine oxalate excretion (180–350 μmol)		
Hyperoxaluria	33	26.2
Normoxaluria	93	73.8
Urine citrate excretion (2.4–5.1 mmol)		
Hypocitraturia	18	14.3
Normocitraturia	108	85.7
Stone number		
Multiple	87	69.0
Single	39	31.0
Stone frequency		
Primary	31	24.6
Recurrence	95	75.4
Stone component		
CaOx (>50%)	107	84.9
CaP (>50%)	19	15.1

[a] <7.5 mmol in male, <6.3 mmol in female, or <0.1 mmol/kg.

citrate were measured. Patients were diagnosed as hypercalciuric when 24-h urinary calcium excretion was greater than 7.5 mmol in male patients or 6.3 mmol in female patients or greater than 0.1 mmol/kg of body weight under a normal diet. Patients were considered hyperoxaluria when 24-h urinary oxalate excretion was greater than 350 μmol and were considered hypocitraturic when 24-h urinary citrate excretion was lower than 2.4 mmol under a normal diet. The number of stones was confirmed by X-ray before treatment. The stone composition was analyzed by infrared spectroscopy. Considering the complex etiology and different pathogenesis, we rigorously eliminated cases of hyperparathyroidism by measurement of serum parathyroid hormone level (>54 pg/ml) and serum calcium level corrected for serum albumin and eliminated cases of infected stone largely composed of magnesium ammonium phosphate ($MgNH_4PO_4$ $6H_2O$) and other non-calcium-salt stones, such as cystine stones and uric-acid stones by stone composition analyses.

The control group consisted of 214 aged Japanese subjects (mean age $\pm$ s.d. $= 68.6 \pm 11.8$ years, 165 males and 49 females) without a history of nephrolithiasis or a family history of stone disease. All of these control subjects were given a general medical inquiry and partly confirmed with X-ray and routine blood and urine assays. All of the patients and controls were recruited from the same racial, ethnic, and the same geographical and environment stratification. The protocol was previously approved by the Institutional Review Boards and the appropriate informed consent was obtained from all patients and control subjects in this study. Genomic DNA from patients and controls was extracted from peripheral blood using a QIAamp Blood kit (Qiagen, Hilden, Germany).

Long-range PCR amplification

The human *OPN* gene maps to the chromosome 4 (4q13), spans about 11 kb and contains seven exons.[39] A long-range polymerase chain reaction (PCR) spanning the entire *OPN* gene sequence was performed on genomic DNA samples from 126 patients and 214 controls, using the platinum *Taq* DNA polymerase high-fidelity kit (Invitrogen, Carlsbad, CA, USA), which possesses a proofreading ability to rule out errors that may occur during the long-range amplification. Briefly, according to the manufacturer's guidelines, 300 ng of genomic DNA was used as a template in 50 μl reactions. Specific amplification primers were designed from published sequences of human *OPN* (GenBank accession no. D14813) and are listed in Table 6. The final concentrations of reagents included 2 mM $MgSO_4$, 0.2 mM deoxynucleoside triphosphate, and 0.2 μM of each primer, 2.5 U high-fidelity platinum *Taq* DNA polymerase and 10-fold diluted high-fidelity PCR buffer. PCR amplification was performed with one cycle at 94°C for 30 s and 35 cycles at 94°C for 30 s, 55°C for 30 s and 68°C for 11 min. Products were resolved on 1% agarose gels and purified with GFX PCR DNA and a gel band purification kit (Amersham Biosciences, Poole, UK).

Genotyping

The *OPN* gene was entirely resequenced with cycle-sequencing PCR amplifications in 45 kidney stone cases and 54 controls. Twenty-five walking primers were designed to cover the entire *OPN* gene using the Primer Premier 5.0 program (Table 6). PCR reactions were performed using the Applied Biosystems (ABI; Foster City, CA, USA) BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) according to the manufacturer's guidelines. Purified long-range PCR products (50 ng) were used in each cycle-sequencing PCR reaction. The cycle-sequencing parameters were

Table 6 | Primers used for long PCR amplification and cycle sequence PCR in the *OPN* gene

	Position		Primer
Long PCR	−2384	Forward	5′-ACCTCCCCGATAGTCAGAAT-3′
	9557	Reverse	5′-AACTTGGTCATGCCCTTGGAT-3′
Sequence PCR			
1	−2304	Forward	5′-TCAACTCCAAAGCATCCCAGAC-3′
2	−1954	Forward	5′-CTTTTTTCAGAAATGCTGCC-3′
3	−1506	Forward	5′-AATGTTCATGAAACCTATG-3′
4	−1066	Forward	5′-CTTTACATACCTTGGGAG-3′
5	−614	Forward	5′-CTCTCAGTCAGAAACTGC-3′
6	−131	Forward	5′-AACCAGAGGGGGAAG TGTGG-3′
7	291	Forward	5′-ACTTAAAAATACTTCCACTGG-3′
8	739	Forward	5′-GTACATGTTGGAAAAATGG-3′
9	1180	Forward	5′-CACTACCATGAGAATTGC-3′
10	1623	Forward	5′-TATGATGAGTTATCGCATG-3′
11	2051	Forward	5′-GTTCAATTCCAGTTGAACAG-3′
12	2476	Forward	5′-GTTAGGATTACTGGGTGGT-3′
13	2938	Forward	5′-CTGGGATTACAGGCGTAGC-3′
14	3387	Forward	5′-CATATGGCTATTGAGCACTC-3′
15	3834	Forward	5′-CTTCTTGTTCCATGGACTG-3′
16	4269	Forward	5′-TTTTTCCATTCATCCCTAC-3′
17	4711	Forward	5′-ACAAGAGGTAAGTTCTCAT-3′
18	5172	Forward	5′-AGCAAGTTAGATAAATTACC-3′
19	5623	Forward	5′-GCCATGGTATGTACTGTG-3′
20	6064	Forward	5′-GCAGACCTGACATCCAGG-3′
21	6494	Forward	5′-CTAACAATTCACTTCCTCT-3′
22	6943	Forward	5′-CCACAGCCACAAGCAGTC-3′
23	7385	Forward	5′-TGTCTATGTTCATTCTATAG-3′
24	7834	Forward	5′-CTTGTCATTTATTTGTGCTG-3′
25	8294	Forward	5′-TGGGCACAAATCTGAATAG-3′

PCR, polymerase chain reaction.

25 cycles of 95°C for 15 s, 55°C for 15 s and 60°C for 4 min. Reaction products were precipitated with ethanol, resuspended in formamide-loading buffer, and electrophoresed on an ABI 3100 automated DNA sequencer (Applied Biosystems). Sequences were aligned to compare with the published genomic sequences (GenBank accession no. D14813) among all individuals and SNPs were validated using SeqScape V2.1 if the second mixed bases were $\geqslant$45% of the highest peak. Four htSNPs were further genotyped in the additional 81 nephrolithiasis cases and 160 controls. Of them, two novel htSNPs, SNP10 and SNP11, were genotyped by direct sequencing, and two htSNPs, rs2853749 and rs2853750, were genotyped by a fluorescent 5′endonuclease assay using an ABI Prism 7700 sequence-detection system, with the recommended protocols. Primers and probes were designed by Assays-by-Design (order no. 185315362, Applied Biosystems). In brief, real-time PCR was performed in a total volume of 25 μl, containing 10–20 ng of genomic DNA, 12.5 μl of 2 × TaqMan universal PCR master mix, 200 nM of forward primer, 200 nM of reverse primer, and 250 nM of TaqMan probe. The amplification reaction was carried out with one cycle at 95°C for 10 min and 40 cycles at 92°C for 15 s, and 60°C for 1 min.

Statistical analysis

The Hardy–Weinberg equilibrium test was done for each polymorphism. The Haploview software package (http://www.broad.mit.edu/mpg/haploview/about.php) was used to estimate the pairwise LD and haplotype blocks structure and identify htSNPs using the CI algorithm.[40] The allele distribution of haplotypes and each

SNP with a minor allele frequency greater than 1% were assessed using χ^2 test or Fisher's exact test between the case and control samples. OR and 95% CI were calculated to determine the risk of kidney stone associated with a given *OPN* allele. Transcription factor-binding sites of the promoter region of *OPN* gene were searched using the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html) and NCBI database (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=506341). Statistical analysis was performed using the statistical package for social sciences (version 10.0; SPSS Inc., Chicago, IL, USA). $P \leqslant 0.05$ was considered as significant.

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